STAIN TECHNOLOGY

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PROGRESS IN THE STANDARDIZATION OF BIOLOGICAL STAINS

TECHNIC OF THE CELLOIDIN METHOD

Attention is called to the paper comprising the greater part of this issue (pp. 37 to 62) by Dr. Wetmore on the Use of the Celloidin Method in Botanical Technic. This paper is primarily a resumé of methods that have been described in various places and at various times, compiling them into a workable procedure to be followed in sectioning and staining after the use of celloidin as an embedding medium. Few new methods are given; but the writer's experience with the technic during a period of many years, makes his compilation of old methods unusually valuable.

It is planned to have this paper by Dr. Wetmore only the first of a series of similar articles dealing with certain phases of histological and cytological technic. As it is anticipated that these papers may be of use for instruction purposes in laboratories where these lines of technic are taught, the Stain Commission plans to put reprints of each of these papers on sale immediately after its publication in STAIN TECHNOLOGY.

The price of the paper by Dr. Wetmore in this issue is 35 cents. It may be ordered either of the Biological Stain Commission, care H. J. Conn, Lock Box 299, Geneva, N. Y., or of the Wistar Institute, University of Pennsylvania, Philadelphia, Pa.

STAINS MARKETED IN ACCURATELY WEIGHED QUANTITIES

Tablet Stains. A matter of some interest to users of biological stains is the fact that certified stains have recently been put on the market in tablet form. An advertisement of these stain tablets occurs elsewhere in this issue. Up to the present time the manufacturers of these tablets have prepared Wright stain, methylene blue, basic fuchsin, gentian violet, and eosin Y in this form.

The tablets are so adjusted in their weight that each one contains 0.1 g. of *actual dye*. Thus in the case of eosin Y the tablets now on the market have been found by the Stain Commission to have an average weight of 0.1486 g. (20 tablets weighed); and as the dye con-

tent was found to be 71.2%, a simple calculation shows that the average amount of pure dye in each tablet is 0.106 g. They should, therefore, furnish a very rapid means of permitting the user of stains to prepare a solution of exactly known strength.

Tablets have one slight draw-back, however: they are occasionally damaged in handling, so that they weigh less when used than when they left the tableting machine. Thus, of the twenty eosin tablets above mentioned, one weighed only 0.1167 g. while the other nineteen varied only from 0.1431 to 0.1574 g. Accordingly any tablet should be examined critically to be sure it is perfect before using, if it is desired to have a solution of accurate strength. If this limitation of tablets is remembered, however, the tablet stains should be very useful for the rapid preparation of solutions of reasonable accuracy; and should be particularly valuable in field laboratories where balances are lacking.

Stains in capsules. Still more recently comes the announcement (in another place among the advertising pages of this issue) from a different company, of certified stains put out in capsules, each capsule, we understand, containing exactly 0.5 g. of *pure dye*. The object of these capsule stains is the same as that of the tablet stains, altho the quantity in each individual portion of stain is five times as large. Whether 0.1 g. or 0.5 g. will prove the more convenient size, time alone will tell. The capsules have the same advantage as the tablets when it is desired to make staining solutions without weighing.

Capsules have one advantage over tablets in that mechanical injury does not cause loss of weight to the contents. On the other hand, the capsules are undoubtedly filled by hand, so that a check-up by the Commission on the weights of a set of samples submitted gives no indication as to the variation which can be expected in market samples. The user, in other words, must accept the manufacturer's statement as to the weight of the sample in the capsule. At present writing, since no sample has been submitted, no opportunity has been given the Commission to check up on the matter; but undoubtedly the weights will be correct.

The manufacturers of these capsule stains announce that they will also prepare assortment packages made up of capsules containing any combination of stains requested by the purchaser. They state that certified stains are to be included in all such packages when those dyes ordered by the purchaser have been put on the certification basis. Some statement to that effect will undoubtedly be placed in the package giving the certification number of the stains included. The regular certification label will not be used however, as it is

obvious that the Commission assumes no responsibility for the correct filling of the separate capsules in such a package.

Attention of the readers is called to these capsule and tablet stains. So far as we know it is the first time that certified stains have been put on the market in any such form, except in the very special case of methylene blue tablets for the reductase test of milk.

H. J. Conn.

Stains Recently Certified

In the table below is given a list of the batches of stain approved since the last one listed in the January number of this journal.

Stains Certified December 1, 1931 to February 29, 1932*

Name of dye	Certification No. of batch	Dye Content	Objects of tests made by Commission†	Date approved
Methyl green	NG 9	....	In histology and as component of Pappenheim stain	Dec. 1, 1931
Sudan III	NY 4	86%	As fat stain	Dec. 15, 1931
Methylene blue	LA 3	81%	As histological and bacteriological stain	Dec. 4, 1931
Gentian violet tablets	BB 1	81%	As histological, cytological and bacteriological stain	Dec. 22, 1931
Basic fuchsin tablets	BF 1	83%	As bacteriological stain and as constituent of Endo medium	Dec. 22, 1931
Methylene blue tablets	BA 1	76%	As histological and bacteriological stain	Dec. 22, 1931
Eosin Y tablets	BE 1	88%	As histological stain and as constituent of blood stains	Dec. 22, 1931
Crystal violet	NC 13	95%	As histological, cytological and bacteriological stain	Jan. 11, 1932
Neutral red	NX 5	79%	As histological and vital stain	Jan. 25, 1932
Basic fuchsin	CF 13	72%	As bacteriological stain, for use in Feulgen reaction and in Endo medium	Jan. 25, 1932
Wright stain	CWr 7	....	As blood stain	Feb. 26, 1932

*The name of the company submitting any one of these dyes will be furnished on request.

†It is not to be inferred that these are the only uses for which each of these samples may be employed. The Commission ordinarily tests each dye for such of its common uses as seem to give the most severe check as to its staining value. Certification does not in any instance, however, imply approval for medicinal use.

THE USE OF CELLOIDIN IN BOTANICAL TECHNIC

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Paraffin has long enjoyed the favor of biologists as a medium for embedding plant and animal tissues, thereby making possible the cutting of thin sections and the preparation of permanent slides for microscopic study. There is much to be said for its use. It is inexpensive and convenient. Its use permits speed in obtaining the desired sections, and, if the initial fixation has been good, the resulting slides are often very satisfactory. With plant materials, however, it sometimes happens that the permanent slides obtained from objects embedded in paraffin are less worth while than is desired, even when the preliminary fixation seemed reasonably adequate. Especially is this true of the heterogeneous tissues composing the roots, stems, or leaves of woody plants. In such cases, it would seem as if another embedding medium were suggested. Since the use of collodion by Duval (1879), this medium has received more or less attention. That it is excellent as an embedding matrix has been admitted in many cases. The majority of writers, however, seem divided in opinion concerning its merits or its demerits. Thus Professor Chamberlain (1924) states in the last edition of his "Methods of Plant Histology" (p. 129), "When material is to be embedded, use celloidin as a last resort. Use paraffin when you *can*, celloidin when you *must*" (his italics.) In the seventh edition of Strasburger's "Botanische Practicum" (1923, p. 79) we read, "Die Zelloidineinbettung kommt nur in vereinzelten Fällen in Betracht, ist weit mühsamer und umständlicher und wird nur dort Anwendung finden, wo die Paraffin-Methode versagt." On the other hand, Taylor[1] states that "it is probable that serious attempts to employ celloidin for cytological studies will produce results superior to the paraffin method in reduction of chromosome clumping or retraction of satellites, etc., but the changes must be most gradual and the sections very thin." Because of this varied opinion on the use of celloidin and because of the failure to appreciate the possibility of employing it even for embedding delicate objects, it seems advisable to prepare a statement on the celloidin method with some discussion of its merits in botanical microtechnic. It should be mentioned at this time that the method

[1]See McClung, C. E., et al. (1929.)

as outlined here is that initiated by Professor E. C. Jeffrey (1917) of the Laboratories of Plant Morphology at Harvard University more than two decades ago and generally employed—with changes—since that time by him and his students. (See Plowman, 1904.)

This statement includes a preliminary general discussion of the essentials of the process. It is followed by a more detailed treatment of procedure, with some mention of variations in the technic which may be employed in certain special cases. It is the hope of the writer that a much wider appreciation of the ready applicability of celloidin to special histological problems in botany may result from an adoption of some of the methods contained herein.

Treatment Preliminary to Embedding

In general, the sequence preliminary to embedding in celloidin is the same as that usually followed when paraffin is to be employed. The material must be collected, properly cleaned, and cut into small pieces, the size of the pieces being determined by the nature of the subject. The problems of fixation are not different from those confronting one if paraffin is to be the embedding medium. When the material is fixed and thoroly washed, it is then softened, or bleached, or demineralized, as occasion demands. After the reagents used for these purposes are removed by further washing, the material is dehydrated, generally with increasing percentages of ethyl alcohol. While in 95% alcohol, a thoro pumping of the material is advisable to remove as much air as possible, thereby facilitating the penetration of the celloidin into the material. The complete removal of water is the final step preliminary to impregnation and embedding.

General Statement of the Celloidin Method

Collodion or pyroxylin is a trinitrocellulose derived from cotton. Various patent collodions have appeared on the world market under such names as "celloidin," "parlodion," etc. These are more often spoken of individually or collectively as "celloidin," and the method of embedding in which collodion is used as "the celloidin method." Such usage, however, altho very common and followed in the present paper, has no chemical sanction. Of these collodions obtainable on the American market, Mallinckrodt's pyroxylin, Schering's pyroxyline on which the patent has lapsed, and Dupont's parlodion are the best known. The experience of the writer's laboratory suggests that the former is at present of a more consistent uniformity in going into solution. The pyroxylin comes in ounce bottles of small pieces which are of fairly uniform thickness. These pieces should be

washed with water and then with alcohol to facilitate drying. After being spread out in a flat dish until thoroly dry, they are ready for making solutions. Tho various solvents have been employed from time to time, e. g. acetone, ether-acetone, ether-synthol, etc., ether-alcohol (equal parts of coal tar ether and either 95% or absolute ethyl alcohol) is more generally used for this purpose. In the embedding process, 2%, 4%, 6%, 8% and 10% solutions are necessary and often higher percentages at 2% intervals from 10% to 20% have been employed. Under the methods to be suggested below, these higher percentages, always hard to dissolve and difficult to handle, are not required.

The material to be embedded, after having been pumped and entirely freed of water, is placed in a bottle and covered with 2% celloidin (2% of celloidin in ether-alcohol). If the material is delicate, it may be advisable to pass it thru an intermediate stage of ether-alcohol.

From this point on, the procedures advocated for the employment of celloidin have varied. These variations may readily be classed under two heads,—(a) methods in which the celloidin is used cold; (b) methods in which heat is employed. Earlier workers (and even some present day histologists) utilize the solutions as made,—cold. When so used, either the material is passed from the 2% solution to the 4%, from the 4% to the 6%, and so on, after one to several days in each percentage (the length of time depending on the nature of the material) or else, the cork stopper is removed from the bottle, when the material is in 2%, and the solution is allowed to become more concentrated in this slow fashion thru the evaporation of the solvents. Before the material is thoroly impregnated and embedded, a matter of weeks may well have passed. This prolonged wait for results seems to have been one cause for objection to the use of celloidin.

The second method entails the use of heat. Experience indicates the advisability of using a wide-mouthed bottle for this purpose. In these laboratories, a 2½-ounce bottle (listed as No. 2320, 50 mm. in diameter and 75 mm. high, in A. H. Thomas' catalog) has been found very satisfactory. It has a wide mouth, which permits the easy handling of reasonably large pieces of material, and also facilitates the pouring of the celloidin. The dehydrated material is covered with 2% celloidin, and the cork stopper inserted firmly. As indicated on the accompanying diagram, (Fig. 1), the bottle is then securely closed by passing a piece of No. 19 soft brass wire over the cork and thru a pair of wire lugs, one on either side of the bottle. The ends of the wire are then twisted together, thus firmly holding the cork in

place. As an alternative to this method, clamps such as are also indicated in the above-mentioned drawing have been found very satisfactory. These clamps are made of strap steel of $\frac{1}{8}$ inch thickness. It is well to have two heights of these, thus allowing for variations between old and new corks. The sizes best suited to the bottle that we use are $3\frac{7}{16}$ and $3\frac{3}{8}$ inches outside measurement. The

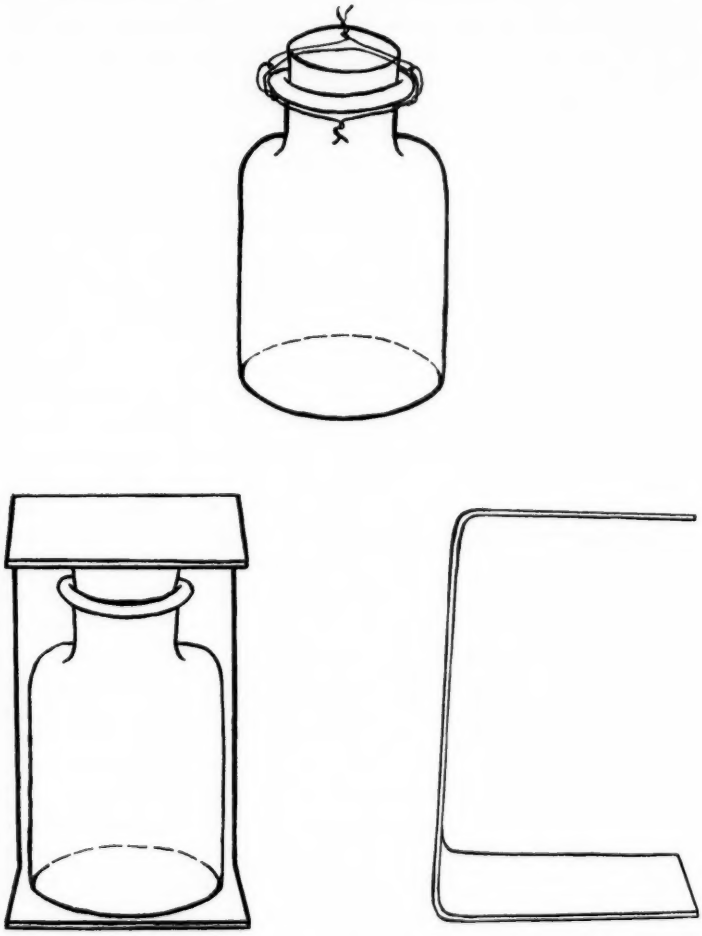

Fig. 1. Methods of securing corks to bottles used in embedding.
Top: Cork secured with wire.
Bottom: Front and side views of special steel clamp used for the same purpose.

writer is indebted to the Department of Botany at Cornell University for the idea of this clamp. It is mentioned in this article with their permission. We have found it very satisfactory and less ruinous to cork stoppers than the older method of wiring the bottles.

When the bottle is securely fastened, it is placed in a constant temperature oven, regulated at about 45° to 55°. To distribute the pressure more generally, it is advisable to lay the bottle on its side. At such a temperature, infiltration is greatly facilitated because of the pressure generated thru the vaporization of the solvents in the bottle. By this means the interval of time necessary before passing on to the

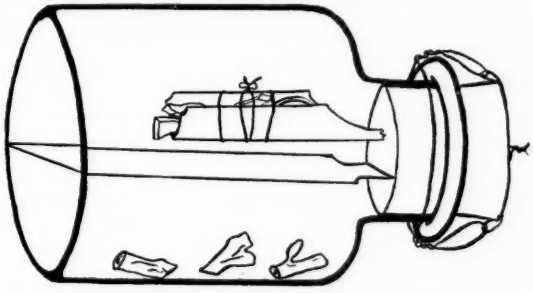

Fig. 2. Embedding bottle with trough to contain fragments of celloidin used for increasing the concentration of the celloidin solution. Modified from Jeffrey (1928).

next percentage is shortened considerably.[2] Thus the change from 2% to 4%, or 4% to 6%, etc., may be made either once or twice a day, due regard being given to the nature of the material.[3] Of course very fleshy or very delicate objects must be moved more slowly. This use of heat removes at once one of the objections offered to the celloidin method.

When 10% celloidin has been reached, it has proven impracticable to change to higher percentages because of the difficulty of pouring the embedding medium and holding back the material which is being embedded. Instead, the celloidin is thickened by the addition of small chips of dry celloidin, which, on going into solution, raise the percentage concentration of the solution. Such a mode of thickening is gradual enough for average botanical subjects. For delicate materials, however, shrinkage may be induced by other than very

[2]A word of caution should be added at this time. Bottles removed from the oven should always be allowed to cool before they are opened to change the mixtures.

[3]It is advisable to discard the 2% after using it once. The subsequent grades of celloidin may be poured into the bottles from which they were taken and used again.

uniform changes. Professor Jeffrey (1928) has devised a scheme which is advantageous in that it is more gradual and almost as fast. A trough is made of zinc or copper as indicated in the drawing. (Fig. 2). This trough is sharpened on one end and the sharp end inserted toward one side of the inner end of the cork. Two or three pieces of celloidin are fastened in this trough with fine wire. When the cork is inserted into the bottle, and the bottle laid on its side, the trough should lie entirely above the surface of the liquid. The ether and alcohol vapors are taken up by the solid celloidin. If the troughs be changed daily, in order that the vapors collected by the pieces of celloidin fastened in them may pass off, the embedding matrix becomes gradually thicker and so gradually that no shrinkage seems to result.

The thickening process is carried on until, when the bottle is quickly inverted, the solution does not flow rapidly around the sides to the lower end of the bottle but rather appears more or less stringy as it runs down. This test should be carried out when the bottles have been cooled. When the celloidin is thickened to the desired degree, the process of infiltration is completed.

To effect the embedding, it is necessary to remove the material from the concentrated celloidin piece by piece with a pair of forceps, and place it in chloroform to harden. It will be found that the piece of material is not readily dropped from the forceps. If the latter be dipped into the chloroform for a moment and removed, the material may then be easily cut free with a scalpel. One should be careful to see that each piece is surrounded by a coat of celloidin so that the material is completely embedded. After 24 hours in chloroform, this reagent is replaced by glycerin-alcohol (equal parts of glycerin and 95% alcohol) in which the embedded material is stored until sectioned. The glycerin-alcohol has a certain softening value so that material left in it for some time is materially improved for sectioning. This general statement of procedure needs amplification in order that the variations needed for different materials may be better understood and followed.

Detailed Statement of Procedure

In view of the general concept that celloidin is an embedding medium for refractory materials to be used when other methods have failed, the extent of the possibilities of the celloidin technic have seldom been realized. The experience of the writer's laboratory suggests that admirable results may be obtained with delicate materials as well as with those harder, heterogeneous tissues comprising the

vegetative parts of woody plants. Thus celloidin seems to give equally as satisfactory results as paraffin for cytological studies of mitosis in root-tips or for investigations of meiosis in developing sporogenous materials. It provides a desirable embedding medium for such delicate subjects as algae or fungi, no shrinkage ordinarily occurring during the infiltration, providing the fixation has been

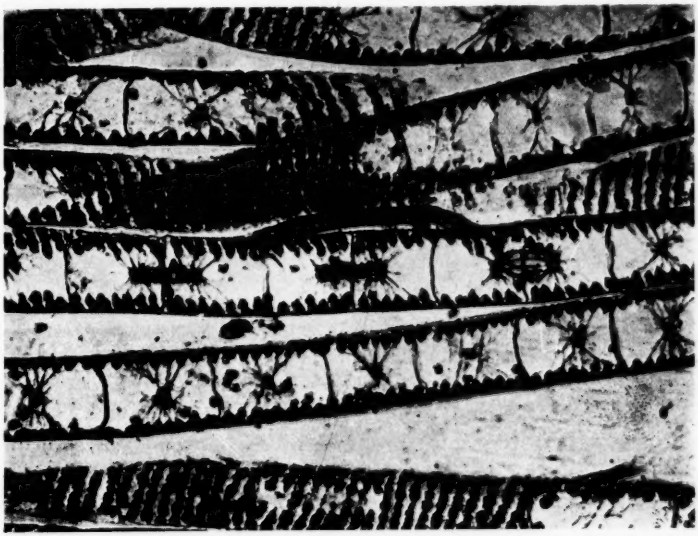

Fig. 3. Mitosis in *Spirogyra*. Material fixed in chrom-acetic mixture, embedded in celloidin, sectioned 5μ thick, stained in Heidenhain's iron-alum hematoxylin, and photographed with an 8 mm. Leitz dry objective and a Homal No. 1 ocular. Photomicrograph taken on a Cramer Spectrum Process plate through a light red filter. Magnification about 300 $\times$.

adequate. Thus the photomicrograph of *Spirogyra* (Fig. 3) during cell division indicates the quality of the results which can be obtained from sections of embedded filaments of *Spirogyra crassa*. That of a portion of a section of a root-tip of *Allium* (Fig. 4) suggests what can be done with chromosomes. These pictures are two from many that have been taken in the laboratory of Professor Jeffrey. Work in his and the writer's laboratories show that such materials as *Fucus*, *Laminaria*, *Chondrus*, *Desmarestia*, *Corallina*, etc., as representative algae of the different groups can be embedded in celloidin in a thoroly satisfactory fashion where due care has been taken with the preliminary fixation. The fruiting bodies of a variety of As-

comycetes and Basidiomycetes have offered no unusual difficulties when subjected to the method, and it proves an excellent means of sectioning plant host and fungal parasite so that the intimate relationships of the association may be studied by differential stains.

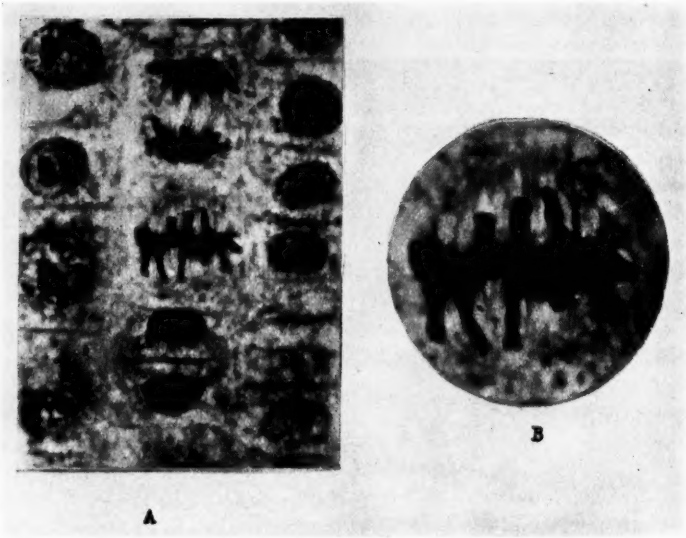

Fig. 4.
A. Mitosis in Root Tip of *Allium*.
B. Central figure of A shown in greater magnification.
Material fixed in chrom-osmo-acetic mixture, embedded in celloidin, sectioned 5μ thick, stained in Heidenhain's iron-alum hematoxylin, and photographed with a 1/7 a Leitz fluorite objective and a No. 3 Homal ocular. Photomicrograph taken on a Cramer Spectrum Process plate through a Cramer light red filter. Magnification in A about 2000×; in B, about 4500×.

For the study of woody tissues and such difficult materials as fossil woods, coal, dried woods, etc., celloidin seems to be the only embedding medium which will give the desired sections, when embedding is necessary. Some of these harder materials may be sectioned without any infiltration at all if they are properly softened. Especially is this true when they are of somewhat uniform texture. When, however, the material is of a heterogeneous nature, as is the case where sections of the entire stem or root are needed, or when it tends to fragment on sectioning as fossil materials or coal often do, embedding proves not only advisable but necessary.

The two criticisms most frequently offered of the celloidin method are (1) that one must wait a considerable time for results; and (2) that thin sections are not readily obtainable. As indicated above, the first objection is in part offset by employing the hot method of infiltration. The second objection is perhaps legitimate, for the beginner. If one wants sections from 1 to 5μ thick, certainly one must have an excellent knife and must use more care than when cutting paraffin ribbons of the same thickness. It is, however, possible to get such sections with material embedded by the hot method, whereas with the older methods of concentration of the celloidin by the evaporation of the solvents, it was not, since the celloidin matrix appeared tough and cartilaginous instead of possessing the even texture which one finds when the thickening process is affected as described above. This cartilaginous consistency seems to result from the differential rates of the evaporation of the ether and alcohol constituting the solvent of the celloidin.

Preparation and Fixation of Material

As indicated above, the preparation of material entails cleaning it thoroly and cutting it up into small enough pieces to insure proper fixation and embedding. The nature of the material itself and the rate of penetration of the fixative will determine the size to be used. It must be recognized, moreover, that material should be cared for, killed and fixed as soon after collection as possible.

Drying or temporary storage is always inimical to satisfactory fixation. Algal and other delicate filamentous materials need no preparation. They should be dropped *at once* into the fixative decided upon. If much water is unavoidably added, it may be advisable to replace the diluted killing and fixing fluid after a time with a new portion. For such material as fungi, or bryophytes, no additional words of caution seem necessary, as long as the pieces are small and are cleaned and freed of undesirable parts.

For the woody or vascular plants, portions of the organs needed are cut into pieces which should not exceed three-eights to one-half inch in length. Fleshy structures such as fleshy rootstocks, the aerial roots of orchids with the velamen, tubers, bulbs, etc., should be divided into proportionately smaller pieces, as both the penetration of the fixative and the infiltration of the celloidin is retarded. Subjects with cutinized or suberized walls or with closely compacted cells require a similar treatment. One should remember that celloidin is essentially an intercellular embedding medium rather than intracellular, as paraffin is. The time necessary to insure proper infil-

tration is determined to a very considerable degree by the extent of the intercellular system. The fleshy parts of the plants must then be cut smaller and be moved more slowly thru the several percentages of celloidin than the vascular and more open tissues of such structures as leaves, bark, etc.

The shapes of the pieces into which the material is cut is determined by the nature of the sections desired. With a homogeneous tissue, it makes little difference how the pieces are cut. With heterogeneous tissues, it is a matter to be considered. Thus with stelar tissues, the blocks are usually cut somewhat wedge-shaped so as to permit the ready obtaining of transverse, radial and tangential sections.

To insure penetration of the fixative and of the celloidin into closely compacted outer cell layers, or thru cutinized or suberized surfaces covering loosely organized internal tissues, it is frequently necessary to make apertures thru the peripheral layers either by direct incisions or by numerous needle pricks. Thus in preparing receptacles of *Fucus* for fixing and embedding, the cutting off of one end of the receptacle or the making of an incision the whole length of it is most effective in insuring good results. In killing and fixing ovaries such as those of *Lilium*, slicing off both ends of the ovary—if too large to leave in one piece, cut into segments—is very effective. Where slow-penetrating fixatives such as osmic acid mixtures are to be utilized, much smaller segments must be planned for. As it is frequently desirable to keep an ovary intact in order that one may cut many sections of ovules in the same stage of development, segments of ovaries are not always desirable. In such cases, one must select a more rapid and better penetrant as a fixing agent. Where cytological studies of microsporogenesis or macrosporogenesis are to be made, it is desirable to lay open the tissues with a razor. Thus the removal of the top of a flower bud down to the desired level may expose either anthers or ovaries permitting more effective penetration of the killing and fixing agents.

Many such cases will arise where the method of preparation of the material must be varied for most effective results. No attempt is made here to cover such cases. Rather a few instances are cited to give an indication of the general type of problem confronting the plant histologist, with indications as to how he meets such situations. Each material offers its own problems and must be so considered.

Fixation

The problems of fixation are deserving of a treatise in themselves. A primary requisite to good embedding is of course good fixation,

whether one use paraffin or celloidin. Inasmuch as excellent discussions of the subject of fixation in general and of botanical materials in particular have been provided in the "Handbook of Microscopical Technique" by McClung (1929) (see Chapter 4, General Botanical Technique, by Taylor; Chapter 9, Fixation and fixatives, by McClung and Allen) as well as in other books on technic and histology, it seems prudent to leave that phase of the preliminary treatment for materials to be embedded for the more comprehensive accounts elsewhere obtainable. Suffice it to say at this time that there is no universal fixative for the celloidin method any more than there is a corresponding preliminary to the paraffin technic. Ordinarily, whatever killing and fixing agents have been found satisfactory for embedding in paraffin will be equally satisfactory for celloidin. Experimentation is the only answer to the problems of fixation.

As has been pointed out by Jeffrey (1928), it is advisable to pump material when it is put into the fixative in order to remove such air as can readily be drawn out, thereby hastening the penetration of the reagents. This can be done in the field by the use of a hand pump which can easily be made from an automobile or a bicycle pump by inverting the plunger and making other related changes thereby altering it so that it acts as a means for exhausting air rather than one for compressing it. The modifications have been described and pictured by Jeffrey so they need no repetition here. If the preparation and fixation of the material is carried out in the laboratory, a pump of the Hyvac type, such as is provided by the Central Scientific Company, is employed. Such a pump becomes an important part of the laboratory equipment.

Washing

Excess of aqueous fixatives should be washed out with water, excess of alcoholic fixatives with the particular percentage of alcohol used as a basis for the fixative. Frequent changes in either case will eventually remove the excess, but where water is called for, a gentle stream of running water is more effective and requires a much shorter time. Various devices to facilitate the washing of numerous bottles at once have been described. That indicated by Parker (1928) is adequate but perhaps more elaborate than is necessary. Chamberlain (1924) has several good suggestions. In the writer's laboratory we employ a washing device, simple in design, comparatively inexpensive, yet very effective. It consists of a piece of ¾ inch brass tubing (Fig. 5) onto each end of which is screwed an elbow. One of these elbows is connected by a nipple to a union which is attached to the faucet. The

other elbow is joined to a second piece of tubing of a length that, when capped, and when placed on the front edge of the sink, keeps the main tube of the device level, thus supporting part of the weight. The length of this main piece of tubing is determined by the width of

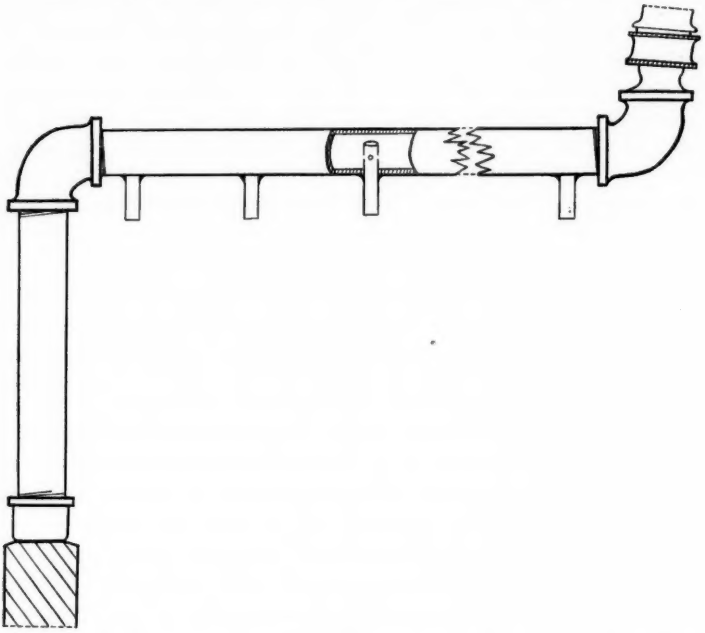

Fig. 5. Washing device, consisting of ¾-inch brass tubing attached to a faucet, with a series of outlets made of ¼-inch tubing.

the sink. In it holes are made about 2 inches apart—the distance to be determined by the size of the bottle ordinarily in use in the laboratory. Into these holes are soldered offsets about ¼ inch in diameter. These offsets however penetrate the tube to a height of at least half the diameter of the tube as indicated in the drawing where a section of the side wall is removed. Such an arrangement establishes a head of water in the tube. Without it, it was found that the offsets did not eject streams of water of equal strength. Those nearest the faucet gave strong sprays of water, when those farthest away provided little or none at all. With the tube full of water and with holes bored in the top of each offset about ¼ inch from the top, it forces streams of water of equal intensity to flow from all of the tubes, a very desirable result.

Over the end of each of the offsets is placed one end of a piece of flexible, thin-walled, rubber tubing, and into the lower end of each of these pieces of tubing is inserted the glass part of a medicine dropper. Each glass tube, placed deep down into the material to be washed, insures a stream of water circulating around the material and provides effective washing easily and quickly. Small pieces, which would be washed out of the bottle by the water, are retained by covering the top of the bottle with cheese cloth held on with an elastic band around the neck of the bottle. The glass tube is pushed thru the cheese cloth well down to the bottom of the bottle. This device has the advantage of occupying a minimal amount of sink room, is comparatively cheap to make, gives a uniform flow of water irrespective of the number of holes, and is simple.

Softening, Demineralization, Etc.

Whatever treatment is suggested for softening, removal of minera deposits from the cell walls, decalcification of crystals, etc., in order that sectioning may be facilitated later, should be carried out at this time, immediately following fixation and washing. Hydrofluoric acid has formed the basis for much of this treatment. This acid, obtainable in 10 pound lead jugs from the J. T. Baker Chemical Co., is reasonably inexpensive if the commercial grade be called for. This grade seems to be as effective as that labelled "Chemically Pure." As indicated by Jeffrey (1928) this acid may be used in aqueous or alcoholic solution. Where the subject to be demineralized or softened is very hard and fairly homogeneous, a pure or nearly pure acid of the above type is to be recommended. Thus oak wood, ebony, mahogany, cocoanut shells, date stones, etc., all can advantageously be placed in the undiluted acid and left until sufficiently soft. In such cases it may be found necessary to replace the acid at intervals with a fresh supply. Even then, weeks of treatment are required for very hard subjects. Less hard materials however should be given a less rigorous exposure, using an acid of 10% to 50% concentration according to the material. Examples such as pine wood, sections of stems of *Tilia* or *Liriodendron*, etc., come in this category. Where the objects to be treated are more heterogeneous, with much parenchyma and at the same time having very tough, wire-like bundles, as in palm stems or cactus stems, or with strands of sclerenchyma as in the rhizomes of many ferns, the use of *aqueous* solutions often leads to undue maceration of the softer tissues before the harder parts are sufficiently acted upon to cut readily. Jeffrey (1928) suggests alcoholic solutions of the acid for such objects, the percentage of alcohol, 50% to 95%,

depending upon their nature. With corn stems, a solution of once-used acid in 95% alcohol, or a 25% to 40% fresh acid in the same strength of alcohol proves to give very satisfactory results in about a week. Palm stems will stand a higher percentage of acid. Hemenway (1930) speaks favorably of his experiences with this treatment.

An alternative method for such heterogeneous tissues, one having the additional value of bleaching at the same time, is to use the proprietary fluid "Diaphanol" (chloridioxide-acetic acid), marketed by E. Leitz., Inc. This fluid, originally employed by E. Schmidt et al. (1921) for technical purposes, and applied by Duysen (1922) to botanical studies seems to have a place in histological technic. The writer's experience with it indicates it to be faster than solutions of hydrofluoric acid and producing no signs of maceration at all. The material is placed in pure diaphanol after it is dehydrated up to 65% alcohol. The bleaching is effected within the first few hours after immersing the pieces of material in the fluid. Palm stems seem to cut more easily and give more satisfactory sections after treatment with this fluid than when treated with alcoholic hydrofluoric acid. The effective softening and bleaching of rootstocks of ferns, of very dark barks, and of necrotic woods is impressive. At present, the outstanding objection is to the price at which it is offered by E. Leitz, Inc., who hold the rights of distribution. If this reagent can be provided for the market at a reasonable figure, it ought to be an excellent substitute for hydrofluoric acid, having the particular advantage over the latter of being used in glass bottles instead of waxed bottles. It is harmless to the hands, moreover, something to be appreciated by one who has worked with hydrofluoric acid to any extent. Personal communications from Professor Wm. R. Taylor and Professor A. F. Hemenway, who have employed this reagent, indicate satisfaction in its usage and an intention to employ it further.

Hard and dry materials, e. g. air-dried wood, the stony or harder layers of certain fruits, (peach stones, cocoanut shells, vegetable ivory, etc.) coal, etc., are badly macerated by the prolonged treatment in hydrofluoric acid necessary for their softening. Even alcoholic solutions of this reagent do not completely solve the problems introduced by these materials, for here a great deal of time is necessary to get results, often weeks or even months. In overcoming this, Jeffrey (1928) advocated the use of a dental vulcanizer. The material is placed in brass tubes, prepared as indicated in the article of Jeffrey indicated above. The writer has found that a tube larger than the one described here can be used in a vulcanizer of No. 3 size (Buffalo Dental Supply Company). The materials in these tubes are covered

with water or some percentage of alcohol, determined in part by the nature of the material and in part by experience. For softer objects, use higher percentages of alcohol; for harder objects, employ more water, i. e. a lower percentage of alcohol. The more alcohol used in the mixture, however, the less the chance of maceration, tho it takes a little more time to effect the softening. The tubes are sealed by screwing up the cap very tightly in a vise or with a pair of stilson wrenches, one working against the other. A tight joint is insured by placing a wadding of asbestos fiber or a thin layer of cork in the cover of the tube before screwing it up. The vulcanizer is then set up according to directions and the temperature run up to 320° C. The time for the hydration of the cell walls at the temperature and pressure within the vulcanizer depends upon the material. Softer materials may take 30 minutes to 1 hour; harder materials may need from one to several hours, again depending upon the nature of the material and the percentage of alcohol in the tube. The slower hydration accompanying the usage of higher percentages of alcohol seems to give more desirable results. After the material has been removed from the vulcanizer—following directions provided with the instrument—and found satisfactorily softened, it is then given a short treatment with hydrofluoric acid to bring about demineralization. A few days is adequate for this purpose.

Other methods have been employed for both hydration and demineralization. Prolonged boiling of *dry* woods is advocated as preliminary treatment in any technic preceding embedding. The use of other acids than hydrofluoric seems unnecessary inasmuch as desilicification demands the use of this reagent and it is also effective in removing other minerals as well. The possibilities of hydrofluoric acid and diaphanol would seem to take care of any situation.

The method described by Crowell (1930) for cutting pieces of dry wood, etc., without further preliminary treatment than to project a jet of live steam on the wood while it is mounted in the object carrier of the microtome, permits one to get sections at once. Comparative studies of sections of a wide range of materials made by the above method with those obtained from portions of the same specimens embedded in celloidin have been made by Mr. Crowell himself in these laboratories. The results indicate the decided superiority of the celloidin sections; yet the Crowell method has a certain value, in that it enables one to make a quick examination of a piece of wood or other hard material, if such is needed for diagnostic or other purposes.

Bleaching

The use of diaphanol as a bleaching agent has already been referred to. It has proved effective in decolorizing tannins and resiniparous deposits so that bark and dark sclerenchyma can readily be cleared and thereafter be stained much more satisfactorily without the necessity of bleaching the sections. Jeffrey and others (see Wetmore, 1926) used various concentrations of a saturated solution of sodium chlorate in concentrated hydrofluoric acid for bleaching. The bleaching with this reagent is not as rapid as with diaphanol, and experience indicates that a frequent result is to make the material very brittle and difficult to section. If used carefully, however, excellent results may be obtained.

The use of any reagent to soften, demineralize, or bleach demands careful washing afterwards. Running water is desired for this purpose for all such reagents except diaphanol, where 65% alcohol is called for.

Dehydration

Dehydration is usually effected by running the material thru a series of alcohols. An hour or so in each of the following is usually sufficient for the average material—10%, 20%, 30%, 40%, 50%, 60% 70%, 95%, and two washes of 100%. Woods and specimens such as pieces of nut shells, etc., may be dehydrated much more rapidly without danger. More delicate objects, e. g. some of the algae, fungi, etc., shrink or even collapse when raised at 5% intervals. In such instances, it seems that the glycerin method of dehydration, as suggested by Chamberlain (1924) in his description of the Venetian turpentine method, is worth trying. This method is mentioned by Hill (1916) and Hemenway (1930). It has been used in the writer's laboratories for some time. It is much more gradual and less likely to injure the material than alcohol. After washing with water, the procedure is to cover the material with a 10% (approx.) solution of glycerin in water. The stopper of the bottle is removed and slow evaporation is allowed to take place. After the first day or so, the bottle is placed on the top of an oven so that the water may pass off more rapidly. When in pure glycerin, dehydration is completed, and so gradually that no deleterious results are apparent.

The Butyl Alcohol Method of Dehydration. It should be mentioned that ethyl alcohol hardens woody materials. Thus many specimens are harder in 100% ethyl alcohol, ready for embedding, than when they were collected. Zirkle (1930) reports experimenting with other alcohols and follows Mlle. Larbaud (1921) in adopting mixtures of

ethyl and butyl alcohol. These prove more satisfactory in that they maintain the original firmness while they avoid the hardening effects of ethyl alcohol when used alone. Thus many woods, formerly of necessity embedded in celloidin, may be satisfactorily impregnated with and sectioned in paraffin if one so desires. In fact, many woods may be cut without embedding if this method be employed.

Zirkle's program is as follows for specimens containing cambium and phloem along with the xylem—(indicated as percentages)—

Water	95	89	82	70	50	30	15	5	0	0	0
Ethyl alcohol	5	11	18	30	40	50	50	40	25	0	0
Butyl alcohol	0	0	0	0	10	20	35	55	75	100	100

One hour in each is satisfactory except for the final change in 100% butyl alcohol, where it should be left over night.

The procedure given above has proven as satisfactory as a preliminary to embedding in celloidin as in paraffin.

Embedding of Materials

It has long been recognized that many plant materials, sufficiently uniform and adequately firm, can be cut without any supporting embedding medium whatsoever, when they have been properly softened and demineralized. Many woods may be so sectioned. The routine of preliminary treatment is the same as tho the materials were to be embedded. Once dehydrated, the blocks should be stored in glycerin-alcohol until the sections are to be made. Exceptions occur however where embedding proves not only desirable but necessary. Thus it is not possible to obtain good sections of *Pinus strobus* in a transverse plane since the lignified walls tear away from the middle lamella unless embedded. Experience alone will determine what these exceptions are. When however the object to be studied microscopically is less uniform thruout, containing xylem, phloem, cortex and bark, or when softer tissues are involved without sufficient supporting tissues, embedding is necessary.

Tho various schemes have been suggested for avoiding the nitrocellulose method or for re-embedding material in paraffin which has already been impregnated with celloidin, experience suggests that where materials of a botanical nature have to be embedded at all, the celloidin method is adequate. Where preliminary results indicate the advisability of impregnating the material with some supporting medium, that medium should be considered which gives less shrinkage and approaches what seems to be the true picture.

The regular procedure for the impregnation with and embedding in celloidin has already been described. There still remains a dis-

cussion of modifications of the method adopted for mass embedding of small objects such as root-tips, small buds, single anthers or ovules, fern prothallia, filamentous algae, aquatic fungi, etc., as in practice in these laboratories. As described by Jeffrey (1928), the method permits of obtaining sections in a definite and predetermined plane since the orientation of the small objects rests entirely in the hands of the investigator.

Jeffrey's description of the method suggests that, after the dehydration of the material, it be placed in glycerin-alcohol for some hours, perhaps over night. The material is then removed to the stage of a dissecting microscope and prepared as desired under the low power. The ovules to be sectioned may then be withdrawn from the ovaries, the scales of the involucre of some compositaceous form may be removed, the root-tips may be sorted or cut into uniform lengths, etc. The objects to be embedded are then placed on a square of heavy paraffined paper in a drop of glycerin-alcohol and spaced as desired. The specimens on this paper are then allowed to stand for a time, preferably in a warm place, in order that the alcohol may evaporate. During this time, unglazed cardboard is cut into squares 15 to 18 mm. on a side. Experience indicates that sections larger than this are not easily handled.

The subsequent treatment in Jeffrey's article referred to above required that the card be covered with a thin layer of warm glycerin-jelly and that the paraffined paper bearing the sections be inverted upon it. When pressed flat by placing a weighted microscopic slide on top of this paper, the glycerin jelly is allowed to set—an hour or more being necessary for this purpose. The slide is then removed and the cardboard squares placed overnight in 95% alcohol. Subsequent pricking of the glycerin jelly with a No. 12 needle, followed by embedding after the usual method described above for celloidin insures infiltration of the material thru the punctures in the glycerin jelly. When sectioned and stained however, the jelly matrix within the surrounding celloidin takes a deep and objectionable stain. In consequence, modifications of the method as now used in Professor Jeffrey's and the writer's laboratory have been adopted which remove this feature of the process. Tho the sections of the material itself may be no better, the slides appear more satisfactory.

In this procedure, the material is dehydrated as desired, whether by the more gradual glycerin method or by the use of alcohol. It is then prepared, as before, in glycerin-alcohol or alcohol, in accordance with the method of dehydration. If in glycerin, the excess of glycerin is removed with filter paper. Cardboard squares are prepared as

before. These are covered with a layer of 4% or 6% celloidin, preferably freshly made. Following a bath of absolute alcohol, the objects are placed in this celloidin. After a short time, while the celloidin becomes very thick thru the evaporation of the solvents, the cardboard squares with the materials on them are wrapped closely in paraffined paper and the whole held in place with strong thread. Pricks or slits should be made in this paper to allow entrance of celloidin. The pieces are then placed in 100% alcohol, left over night, and passed into 2% celloidin. The regular sequence for celloidin follows. During the embedding process, the celloidin on the card goes into solution, but the closely fitting paper holds the objects in position. This modification of the mass method of embedding does equally well for fern prothallia, buds, filamentous algae, or other small objects. It is also satisfactory for root-tips when longitudinal sections are desired. Where transverse sections of the latter are required, Professor Jeffrey has found it convenient to enclose the root-tips in gelatin capsules which are pricked well to permit the inward passage of the celloidin so that they may be embedded while in these containers. When embedded and ready for sectioning, it is easy to remove the capsules and so to have the block of celloidin free for sectioning in any plane desired.

It should be mentioned that these same methods have proven very satisfactory for small objects of zoological nature, e. g., insect pupae, small insects, small worms, sections of larger worms, etc.

Microtome and Knives

To obtain thin sections of harder vegetable tissues and of materials embedded in celloidin, one needs: (1) a sliding microtome, (2) knives of uniform steel, heavier in style than is required for the sectioning of materials embedded in paraffin and (3) backs, hones and strops to insure the maintenance of a keen and smooth edge.

Sharp knives on a good sliding microtome make it possible to cut sections 5 to 10 μ in thickness from unembedded harder plant tissues —these must of course be softened and demineralized where necessary—and even thinner sections from materials embedded in celloidin. This possibility removes another of the objections—the inability to cut thin sections—from those offered to the use of celloidin as an embedding medium.

There have been many types of sliding microtomes available from time to time. At present, it would seem that the Jeffrey-Thomson instrument, manufactured by the Bausch and Lomb Optical Company,

satisfies the most rigid requirements imposed upon it and gives uniform and consistent results without getting out of order. This instrument was modified from the earlier type devised by Professor R. B. Thomson of the University of Toronto thru the efforts of Professor E. C. Jeffrey of Harvard University. A full description of the instru-

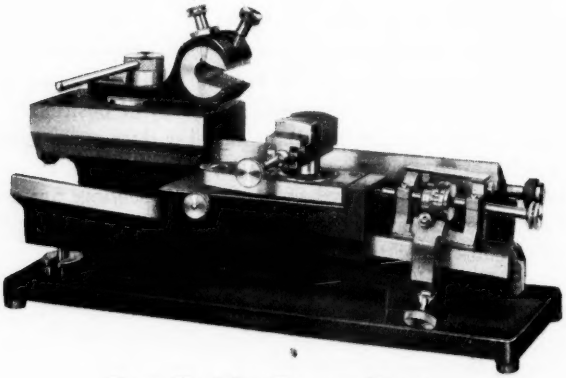

Fig. 6. The Jeffrey-Thompson Microtome.

ment is given by Jeffrey (1928) and in the Bausch and Lomb folder. Figure 6 indicates its general appearance. The experiences of these laboratories over the last few years suggest that the instrument will adequately supply the need for a reasonably priced and efficient microtome where sections of hard tissues or objects embedded in celloidin are desired.

Of the various types of knives tried in these laboratories, none has been found as consistently satisfactory as that produced by R. Jung, Heidelberg, Germany. These knives (Loew Pattern, L Form, No. 247c in the catalog of R. Jung) are now obtainable from the Bausch and Lomb Optical Company.

For sharpening knives to be used with a sliding microtome, one needs a back for each knife in order to insure a reasonably constant bevel to the edge. Honing the knife without a back produces an edge of too thin a nature to stand up to the severe test of cutting hard tissues, whether embedded or unembedded. A back should be purchased for each knife from the manufacturers of the knives, and marked in such a way that it may be kept for use with that knife only. In this way the bevel of the knife edge is maintained at something of a constant angle, an angle which should approximate 23°.

The hones employed should be of the best. Of the many available, the Arkansas oil stones have proven very satisfactory. They can be purchased in different degrees of hardness and of different sizes. Stones 8 inches to 12 inches long are advisable. If a softer stone be employed for rapidly cutting the bad places out of the knife and a harder stone used for smoothing the edge, a little practice soon enables one to obtain the cutting quality one desires.

Various strops are obtainable. We have employed one with four stropping surfaces, obtainable from the R. Jung Company. This company also lists proper pastes for application to these surfaces to permit of the desired quality for the cutting edges. The results fully justify the expense.

Recently more elaborate mechanical devices have appeared on the market for sharpening microtome knives. The Long microtome knife sharpener, devised by Dr. J. A. Long of the University of California, has earned for itself considerable prestige. No hones nor strops are required when this instrument is available. It is said that some care is necessary on the part of the operator to become efficient in the use of the apparatus however. Once accustomed to it, hours of laborious work are saved and a uniform and consistent edge assured for the knives in use.

After a time, knives are worn back so that they become too thick to maintain anything like the desired bevel. In such cases, a good carborundum wheel is very desirable to facilitate regrinding the knives, thereby thinning down the edges to more efficient dimensions. Here again the quality of the results depends upon the experience of the operator.

Sectioning of Materials

Materials embedded in celloidin are ready for sectioning after they have been in glycerin-alcohol for some days. If slightly hard, a longer period in the mixture may be advisable since this reagent has a certain softening value.

A piece of the embedded material is removed from the glycerin-alcohol, trimmed of excess celloidin, and placed at once in the object carrier in a way that will permit of sections being made in a desired plane. Whether the objects be small and embedded individually, or whether they be mounted on cardboard after the mass method of embedding, it is desirable that they should be fixed on a block to raise them to a level such that the knife will readily section them. Blocks of a soft wood, such as white pine, about ¾ inch long and about ½ inch to ¾ inch in cross section should be prepared beforehand. One of these wooden blocks is dipped in newly-made 6%

celloidin (at least not previously used in the embedding process) so that one end is completely wet with it. The block is then allowed to dry after which the end is again immersed as well as the block of material to be mounted. The latter is then placed upon the wet block and the whole allowed to set until dry— a matter of 5 to 10 minutes.

After placing the mounted material, or a larger unmounted piece, in the object carrier and clamping it in tightly, it is ready for sectioning. A sharpened knife is placed in the knife holder, and fastened at an oblique angle. The micrometer screw is set to cut the desired thickness. A good quality camel's hair pencil brush is wet in 95% alcohol and both object and knife edge are kept wet with this liquid. If the object carrier be pushed forward up the inclined plane by the micrometer screw which has been properly adjusted, each stroke of the knife then removes a section of the desired thickness. By keeping the wet brush on the top of the object, the section is floated on to the knife blade from which it is readily removed to a watch glass of 95% alcohol. If the section shows signs of curling, it is well to float it up to a corner of the knife and to leave it there until it begins to dry out before removing it to the watch glass. This will lessen the tendency to curl. Sections in any number may thus be cut. Examination of each or occasional sections under the microscope will determine the quality and nature of the sections.

The angle of the knife to the block of material as well as the pitch of the knife is largely determined by experience. For hard materials, the knife should approach a position at right angles to the motion of the knife carrier. Thus in sectioning embedded pieces of coal, the knife should be kept at right angles, as when sectioning materials embedded in paraffin. The softer the object to be cut, the more obliquely the knife should be placed to the path of movement of the knife carrier.

An objection frequently offered to the celloidin technic is the difficulty of cutting serial sections. To be sure the method of obtaining such sections is slower than when paraffin is used since each section is handled individually. Ribbons are not possible. Various methods have been described. That mentioned by Foster (1926) has been found adequate to our needs, being simple and successful. The sections when cut are placed on a microscopic slide in the order cut, the slide being kept moist with 95% alcohol to hinder the sections from drying out. It is frequently desirable to make a drawing of a microscopic slide and of the intended position of the cover slip on it. If a microscopic slide be placed on this drawing, the sections may be

properly arranged as cut. When the space is filled, the excess of 95% alcohol is blotted off with filter paper. The spaces between the individual sections are then painted in with a 2% or a 4% solution of celloidin and the whole allowed to dry. The thickness of the sections determines what percentage to use. Thinner sections require a 2% solution; thicker sections, a 4% solution. Care should be taken to keep as little of this celloidin as possible from getting on the sections themselves, otherwise curling of the margins may result. A little practice soon eliminates this source of trouble. The slides with the sections on them are then removed to a staining jar containing 95% alcohol and a little chloroform to aid in the hardening process. They should be fully hardened in 30 minutes. With a scalpel or section lifter, the sheet of sections is removed from the slide. It is then ready for storing until ready for use—in 95% alcohol—or for staining, as desired.

Staining of Sections

The technic of staining sections must vary with the desires of the worker and with the tissues being prepared. No additional problems are introduced in the staining of celloidin materials. The sections after cutting are in 95% alcohol. The use of aqueous or alcoholic stains demands respectively that the sections be moved into water or into the proper percentage of alcohol in order that the stains may be applied properly and with the least possible chance of damage to the sections. The celloidin matrix does not seem to impede the penetration of the dyes tho it may be colored itself. It is not ordinarily removed as is paraffin, or, if so, not until a later stage when the sections are undergoing dehydrations, since the margins of the unsupported sections frequently curl.

There are certain combinations of stains which prove very desirable. Thus where lignified or sclerified tissues are involved, it is difficult to imagine more contrastive results than can be obtained with Heidenhain's iron-alum hematoxylin followed by safranin. After mordanting with a 3% solution of iron-alum (ferric ammonium sulfate) for 10 to 15 minutes, the iron-alum is washed off with at least 3 washes of water, care being taken to see that neither of the first 2 remains very long on the sections—change as rapidly as can conveniently be done—in order to remove the excess of the mordant. Without this care, ferric hydroxide may be thrown down in the tissues. The third wash of water should be allowed to stand on the sections for some ten or fifteen minutes before being replaced with distilled water. Into this water are put four to six drops of a one-half percent solution of hematoxylin crystals in water, thoroly oxidized. This stain is allowed

to act until the sections appear a definite blue—from 5 to 15 minutes is usually necessary, depending on the material. The excess stain is then washed off with two washes of water. Into the second of these are put a few drops—usually 4 to 8—of a 1% solution of safranin in 50% alcohol and the material left over night. By the next day, the staining is complete and the sections ready for dehydration and permanent mounting. If in a hurry, the time for the penetration of the safranin stain may be shortened by the placing of the watch glass containing the material on an oven at a comparatively low temperature. It pays, however, to use plenty of time, as the brilliancy of the stains frequently is a reward in itself. This is true with safranin.

Where cytological studies are being pursued and it proves necessary to employ sections rather than the smear methods, overstaining for 24 hours or so with Heidenhain's iron-hematoxylin, followed by destaining with a 10% solution of iron-alum gives excellent results. The chromosomes are black and sharp against a clear cytoplasm if the differentiation is carefully carried out.

Various stains have been found satisfactory for different purposes, irrespective of embedding medium. For references to special combinations, it will be found well to consult Taylor (in McClung, 1929) and Chamberlain (1924).

Dehydration and Mounting of Sections

To dehydrate sections embedded in celloidin preparatory to mounting them permanently on microscopic slides, whether they be sheets of serial sections or individual sections, one needs four watch glasses. Into the first is put 95% alcohol. Into the second and third 100% alcohol is poured, with a few drops of chloroform. This last is needed to offset the solvent effect of the absolute alcohol. Into the fourth, benzol or xylol is added as a clearing agent. If it be desired to remove the celloidin matrix before mounting, this can easily be done by placing a watch glass of ether or ether-alcohol between that containing the second 100% alcohol and that with the clearing agent. When ready, the sections are moved individually with a section lifter or a pair of forceps (if the sections are small) from one watch glass to the next of the series, care being taken to remove the excess liquid from the section by touching it to a piece of filter paper before passing it on to the next reagent. Where anilin dyes are used, it will be found that the excess of stain comes out in the 95% and the first 100% alcohol, both from the matrix and the material itself.

After the sections have cleared sufficiently in benzol or xylol, they are removed to the microscopic slide and arranged as desired. A little

Canada balsam, not too thin, is then placed on the sections and the cover slip is put in place. After 24 hours of drying on a warming table, a lead weight is placed on the slide to press out the excess of balsam, thus insuring the flatness of the sections. The ease with which slides dry out when cleared in benzol and when the balsam is dissolved in benzol, as contrasted with those where xylol has been used, has resulted in the continued use of the former clearing agent in the writer's laboratory. Sections prepared in this way are flat and readily lend themselves to photomicrography.

Application of the Procedure

Variations in this technic as outlined in its essentials here will be suggested to any investigator as new problems present themselves. Much of the adverse criticism one finds to this procedure seems to be centered in the expenditure of time to learn a new technic and of money to provide a new equipment. Experience would seem to suggest that the end would justify the expenditure and trouble. The increase of time over that required for the paraffin method before one can section and study the material under investigation would scarcely be considered worth while in any laboratory if comparable results could be obtained with paraffin. In many instances (e. g. young roots, soft floral and other parts) such can be done. In other instances, however, e. g. in plant tissue containing hard elements, or even in many delicate subjects such as algae, fungi, etc., the present writer feels that the slides obtained from paraffin can scarcely be compared with those obtained from like materials embedded in celloidin. Where results are so distinctly superior, time has no significance. For such reasons familiarity with the technic, on the part of plant histologists, is decidedly advisable.

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HEIDENHAIN'S HEMATOXYLIN USED WITH THE SMEAR TECHNIC

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Abstract.—**Heidenhain's hematoxylin, according to the following formula, may be used for studying chromosomes in temporary smears of pollen mother cells: 1 part 0.5% hematoxylin and 4% iron-alum (equal parts of each); 1 part 95% alcohol; and 2 parts glacial acetic acid.**

The aceto-carmin technic as developed by Belling[1] has been used to great advantage in making chromosome counts from temporary smears of pollen mother cells. It has been found in this laboratory, however, that Heidenhain's hematoxylin may be used for the same purpose and with results superior to the usual aceto-carmin stain.

The stain is made up of 1 part of a mixture of equal parts 0.5% hematoxylin and 4% iron-alum, 1 part 95% alcohol, and 2 parts of glacial acetic acid. The acetic acid is used in this formula because it gives excellent optical differentiation to the preparations. The alcohol not only intensifies the sharpness of the stain by apparently behaving like a mordant, but it also counteracts the swelling action of the acetic acid. It is possible that a slight variation in the amounts of the components of this mixture may be more effective on some material. Experimental variations applied to the pollen mother cells of *Vicia faba* (vetch) and *Callisia repens* indicate, however, that the above formula is the most effective for securing proper fixation and sharpness of stain.

Smears are made in the same manner as in the present aceto-carmin technic. The anthers are removed with a needle and placed in a drop of stain on a slide. They are then smeared with a flat needle; the anther debris is removed with forceps; and the preparation covered with a cover-slip. The drop of stain should spread evenly under the cover; but if an excessive amount be used, the chromosomes tend to stain too darkly.

The chief advantage of the hematoxylin over the aceto-carmin technic is that the meiotic chromosomes are stained immediately and

[1]Belling, J. The iron-aceto-carmin method of fixing and staining chromosomes. Biol. Bul., **50**, 160–162. 1926.

with a sharpness obtainable with the latter only after a period of time, usually 24 hours or more.

Fig. 1. is a photomicrograph presenting two examples of meiotic chromosomes stained by this method. In (A) the bivalent chromosome of *Vicia faba* are shown at the heterotypic metaphase; (B) shows a similar configuration in *Callisia repens*.

It is interesting to note that the long bivalent chromosome of *V. faba*, as found in practically all of the metaphase figures obtained in this work, contains 6 chiasmata. In no case was the number of chiasmata observed to exceed 6, altho in a few cells only 5 were evident. Maeda[2] made a study of the chromosome configurations in this plant and found that the number of chiasmata in the long bivalent varied from 2 to 13, the average number being 8.

Fig. 1. Metaphase configuration of the heterotypic division in pollen mother cells from temporary smears stained with hematoxylin. (A). *Vicia faba*, showing the long bivalent. (B) *Callisia repens*.

The only apparent explanation of the difference between these results and those of Maeda's is offered by a consideration of the time when these plants were studied. This study of the chromosomes of *Vicia faba* was undertaken very late in the fall when the weather was relatively cold. This would seem to suggest that chiasma formation is influenced by temperature, and that the cold weather is responsible for causing a reduction in the number of chiasmata formed in this plant.

[2]Maeda, T. On the configuration of gemini in the pollen mother cells of *Vicia faba*, L. Mem. Coll. Sci. Kyoto Imp. Univ., **5**, ser. B, art. 6. 1930.

ACETONE AS A SUBSTITUTE FOR ALCOHOL IN MICROTECHNIC

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Ethyl alcohol has had such universal acceptance as a reagent in microtechnic that the substitutes suggested from time to time have not had adequate recognition. In recent years, it has become more difficult and sometimes impossible, even in state schools, to obtain grain alcohol. Having had many teachers enrolled in his summer course in microtechnic, the writer has been impressed with the desirability of disseminating information concerning substitutes for ethyl alcohol.

The n-butyl alcohol process[1] is used extensively in this laboratory in studies on the graft union and crowngall (in press). Recently n-propyl alcohol has been proposed as a dehydrating agent[2], while iso-propyl alcohol has been used mixed with methyl alcohol in bacteriology[3]. Acetone is a good dehydrating agent, and has had some applications in biological work[4], but its possibilities, especially as an ingredient of killing formulae, have not been utilized. Several schedules which eliminate alcohols, from the killing as well as dehydrating processes, are presented here as based on specific subjects. Kill small pieces of tomato leaf in the following: acetone 30 cc., formalin U. S. P. 5 cc., glacial acetic acid 5 cc., water 160 cc., $HgCl_2$ to saturation. Pump with an aspirator until the pieces sink. After 36 hours dehydrate in grades of acetone, 15, 20, 25, 30, 35, 40, 50%, etc., thru two changes of anhydrous acetone. For this subject 15 minute intervals suffice. Clear in four grades of acetone-xylol, embed and section. The stain combination illustrated in Fig. 1 is Mayer's hemalum-safranin. In the staining procedure the lower grades of acetone differentiate the safranin, whereas the anhydrous acetone, which replaces absolute alcohol, does not affect the stains, thereby being superior to alcohol. Clear the preparation in cedar oil or carbol-xylol and mount in balsam. Fig. 1 illustrates the quality of the

[1]Zirkle, C. The use of n-butyl alcohol in dehydrating woody tissue for paraffin embedding. Science, **71**, 103–104. 1930.

[2]Sheridan, W. F. Use of (A) normal propyl alcohol and (B) low melting point paraffin in infiltration to prevent distortion and hardening of tissues. J. Tech. Methr. and Bull. Inter. Assn. Med. Museums, **12**, 125–126. 1929.

[3]Conn, H. J. A new substitute for ethyl alcohol in the Gram stain. Stain Tech. 3, 71–72. 1928.

[4]Kopeloff, N., and Cohen, P. Further studies on a modification of the Gram Stain. Stain Techn. **3**, 64. 1928.

Scales, F. M. A new method for differential staining of bacteria. J. Inf. Dis. **31**, 494–8. 1922.

killing, in a very succulent and delicate subject. Note that the palisade cells are well preserved, as shown by the peripherial position of the plastids.

The following procedure preserves the chromatic elements of the nuclei in the root tip of onion, and shows a chromonema-like doubleness in the very early prophase, as well as considerable detail in late telophase. Kill in: acetone 50cc, formalin 7cc., acetic acid 3 cc., water 40 cc. After 12 hours dehydrate in acetone 50, 60, 70% etc., and finish as before. Brilliant staining is obtained with iodine-gentian violet, safranin-fast green, and iron-hematoxylin.

For still more critical results kill the tissues in the following: 1% chromic acid 50 cc., formalin U. S. P. 10 cc., glacial acetic acid 5 cc.,

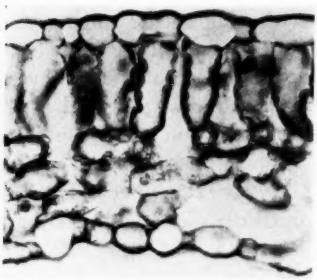

Fig. 1. Leaf of red currant tomato. × 400.

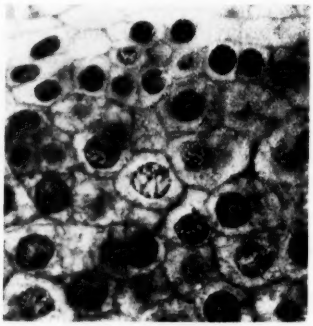

Fig. 2. Microsporocytes of Emperor narcissus. × 400.

water 35 cc. Pump as before. After 3 to 5 days the tissues should be well fixed. Instead of washing in running water as usually prescribed, rinse in 10% formalin and pour the pieces into a liter or more of 10% formalin. Material may be left in this for at least 2 months without injury. This treatment washes by diffusion in 2 to 5 days, without softening the tissues. Dehydrate in grades of acetone; 10, 15, 20% etc., as before. Fig. 2 shows pollen-mother-cells of Narcissus. Note that the cells of the tapetum and the sporocytes are well preserved. Mayer's hemalum, a histological stain, was used, in order to overstain the cell walls and cytoplasm and thereby show that very little plasmolysis occurred. With a nuclear stain the chromomeres of the early prophase spireme are evident.

The above procedures have been used on rusts, smuts, crown gall, general histological work, and with finer gradations in cytological work on Angiosperms and Basidiomycetes. For the field laboratory, where it is unwise to keep ethyl alcohol, or for the school where it is not obtainable, these processes may fill an acute need.

LABORATORY HINTS FROM THE LITERATURE

Under this heading are given interesting new methods in microscopy that have been noticed in other publications. The readers' assistance is urged in calling the editor's attention to any outstanding methods in this field that are overlooked. Thru coöperation with the Wistar Institute of Anatomy and Biology of Philadelphia, these same abstracts are to appear on the Laboratory Service Cards of that institute. Those desiring some of these cards without subscribing for the entire Bibliographic Service of the Wistar Institute, can order direct from STAIN TECHNOLOGY, at a price of 6 cents each.

BAUMGARTNER, W. J. and PAYNE, M. A. 'Intravitam' technic used in studies on the living cells of grasshoppers. J. Exp. Zool., **59**, 359–393. 1931.

This article, altho not mentioning stains in the technic, is here described because of its great interest and value to the cytologist. By means of this method, cellular structure and inclusions are seen in the living cell, connected with the organism, so that its normal metabolism remains unaltered.

A male grasshopper is anaesthetized for several seconds and his hind legs are severed at the autonomous joints. The wings are cut off behind the pronotum, and a rectangular opening is cut thru the chitinous wall of the second, third, and fourth segment, just left of the middorsal line. The insect is then placed on its right side on a slide, parallel to its width. Melted paraffin is run from a pipette over the forelegs and antennae, around the head and along the ventral side of the body, which faces the right hand side of the slide. A narrow ribbon of paraffin is led up and back to the anterior end of the insect. Spiracles on the abdominal wall and the anal aperture are left open. The three paraffin walls together with the body and the enclosed surface of the slide, form a small basin, which is filled with Belar's fluid (Romeis' Taschenbuch, p. 18), which is isotonic with the germ cells. Before the grasshopper recovers, the testes are drawn out of the abdominal aperture. The yellowish membrane, which encloses the follicles, is carefully torn away, and the latter float out in the basin, remaining attached to the vasa efferentia. Several follicles are secured with a loop of silk thread, and the latter is attached to paraffin. This facilitates observation of the living cells and allows the study of movements. *Chortophaga viridifasciata* was the species mainly employed, but also other Orthoptera were used, and 2 species of Coleoptera.

With the present scepticism among numerous biologists in interpreting fixed preparations, the following sentences will sound very encouraging for microscopic workers: "Most of the details of spermatogenesis described in fixed tissues have been seen in living cells thru cyst and follicular walls. We have seen the tridimensional nature of the follicle, the loci and variation in the shape of the cysts, growth stages of spermatogonia and spermatids, division stages of cells and transitional stages of spermatids, have seen chromosomes move and divide, have watched them in various stages of formation, and have observed the spindle with mantle fibers and astral rays, the clear, refractive centrosome, the Nebenkern of spermatids, the changing shape of the nucleus, the cytoplasmic droplets on the axial filament."

BOWMAN, P. W. and LINCH, M. N. A short method for the preparation of animal tissues for staining. Science, **74**, 440. 1931.

Excellent slides have been made by means of the following method. Cut the tissue in pieces from 2 to 4 mm. in thickness. Drop these in 10% formalin and heat to boiling. Transfer them to acetone for dehydration and leave them in this liquid for one hour, during which time the acetone should be poured off and renewed with fresh 2 to 3 times. The dish should be covered and put in the paraffin oven, as diffusion takes place more rapidly at higher temperatures. Remove the tissue from the acetone and drop it into a container of melted paraffin. Leave this in the oven for an hour.

Embed, mount and cut in the usual manner. Smear the slides lightly with Mayer's albumen, float the sections over this on a drop of water, warm gently to expand them and drain off the excess. Lay the slides flat in the paraffin oven. At the end of one hour remove them. After they have cooled, put them into xylol to dissolve the paraffin and proceed in the usual way to stain and finish them.

BRODERS, A. C. Modification of Wilson's fresh frozen section technic. J. Lab. & Clin. Med., **16**, 734–738. 1931.

In this procedure tap water is used instead of the dextrin, physiologic salt solution and glucose. Slides are ready for examination in 50 seconds.

Prepare slices of tissue of 2mm., to 10mm., in diameter. Dip in tap water and place to the best advantage in the freezing microtome, remembering that the cutenous surface should be closest to the knife. If the piece is very small, freeze a drop of water under it to avoid direct contact. Turn on the carbon dioxide in intermittent spurts until about half of the tissue is frozen, and cut immediately above the frozen part. Keep the knife wet and cut sections about 15μ thick. When good sections appear, remove them from the blade with the wet tip of an index finger and transfer to a Pyrex dish with water. Paint the bottom of the dish black, or keep a piece of carbon paper under it. The sections flatten out on the surface and can readily be seen. Insert the end of a large glass rod under a section and carefully raise it. Transfer to Terry's neutral polychrome methylene blue and let them float off. Pick up and float again. Penetration is very rapid. Transfer to tap water, lift once or twice, and let them float until the excess stain washes out. Transfer to fresh water in a bird bath. Mount in water, by the rolling method, or by taking advantage of the surface tension and examine.

COOPER, D. C. Microsporogenesis in *Buginvillaea glabra*. Am. J. Bot., **18**, 337–358. 1931.

A new stain combination was used in these studies. Preparations of microsporogenesis were made as follows: Carnoy's fluid for 20 to 30 seconds, to act as a "sinker", and then fixation in Flemming's solution, or Taylor's modification of Flemming's; other fixatives used were Carnoy's alone, Benda's and Bouin's fluids. Beside the customary Heidenhain's hematoxylin and Flemming's triple stains, another combination has been worked out, of which the author states: "A particularly useful combination of stains was found to be a 1% aqueous solution of methyl green, a 1% aqueous solution of acid fuchsin, and a 1% alcoholic solution of erythrosin. The sections were stained in the methyl green for 1 hour, counterstained in acid fuchsin for 1 minute, and then stained in erythrosin for from 2 to 3 seconds. With this stain, the chromatic granules and the nucleoli in the early prophases were stained green, and the linin fibers were stained red. In the later stages the chromosomes were stained a brilliant green".

CUNNINGHAM, R. S. and TOMPKINS, E. H. The supravital staining of normal human blood cells. Folia Haematologica, **42**, 257–270. 1930.

In physiologically active blood cells mitochondria are stained by Janus green, while specific granules of the polymorphonuclear granulocytes as well as vacuoles of the monocytes, lymphocytes and other cells, if present, are stained by neutral red. By this simple procedure observations of the motility can also be made and small amounts of hemoglobin observed.

Prepare saturated solutions of neutral red (Grübler's "nach Ehrlich für injektion"), and of Janus green (National Aniline "Diazine green"), in absolute alcohol, and keep in a dark place in glass-stoppered bottles. For use add 20 to 30 drops of the concentrated solution from the top of the bottle to 10 cc. of absolute alcohol, the exact number of drops to be determined for each batch. For staining leucemic blood, exudates, and tissue scrapings double the strength of neutral red. For routine slides 3 drops of concentrated Janus green are added to 1 cc. of the dilute neutral red solution; but Janus green somewhat inhibits the staining with neutral red.

Flood perfectly clean slides with the dilute stain, drain off, place the slide in a box, allow to dry, then mark the right side. Place a drop of blood on a cover glass, and transfer to the slide. After the drop has spread, smear the rim with immersion oil or with vaseline of a slightly higher melting point than the temperature of the box enclosing the microscope which should be around 38° C. The slides should remain in darkness, except when studied.

ECKERT, F. Erfahrungen mit der Präparationen von Algen. Mikroskopie f. Naturfreunde **9**, 123–126. 1931.

Upon examination of various algal preparations mounted years ago, the author concludes that glycerin is the most suitable medium. As to fixatives, Pfeiffer's solution (equal volumes of acetic acid, prepared from wood, formalin, and methyl alcohol) is recommended for Chlorophyceae and Batrachospermium, and formalin, 4 to 10%, or

preferably formalin vapor for Cyanophyceae. Both fixatives are for the preservation in natural colors. For the staining of Chlorophyceae use Flemming's solution (chromic acid, 0.7 g.; H_2O, 160 cc.; glacial acetic 5cc.), for 10 to 20 hours. Wash and stain with Delafield's hematoxylin, counterstain with alcoholic safranin, and wash. The nuclei should appear blue-black and the cytoplasm red. Transfer material to glycerin diluted 1 to 10 on a slide; after this has evaporated, add a drop of concentrated glycerin, and cover with cover-glass. Apply "Verschlusslack" in three spots to keep the cover from sliding. After 24 hours ring with Japanese microscopic lake, (see Migula, Der Lackring, Mikr. f. Naturfr. **4**, 276) With neutral glycerin such preparations keep for decades.

Copper-lactophenol (Culactol) is an excellent medium for unstained algae, with the preservation of the green color. This is prepared by mixing 5 g. lactophenol, (phenol 20 g.; lactic acid, *spec. grav. 1.21*, 20 g.; glycerin, *spec, grav. 1.25*, 40 g.; H_2O 20 g.) with 95 cc. H_2O; 0.2 g. copper chloride; and 0.2 g. copper acetate. Use 5 parts of Pfeiffer's fluid to 1 part of culactol, or 6 parts of formalin to 1 part of culactol. These fixatives counteract the shrinking effect of culactol when used alone. For best fixation treat with Pfeiffer's solution for 3 hours, wash and transfer to culactol for 1 day. Mount in glycerin with 10% culactol.

The quality of other media observed is given in a descending order: glycerin jelly, Fehlmann's medium, Hoyer's and Regel's.

FAVORSKY, B. A. Eine modifikation des Silberimpregnationsverfahrens Ramon y Cajals für das periphere Nervensystem. Anat. Anz., **70**, 376–378. 1930.

The following modification of Cajal's silver impregnation method is recommended: immerse tissues in 0.5 to 5% solution of acetic acid, in 50% 60% or 80% alcohols, for 24 hours. For tough tissues use lower percentage of alcohol (50%) and higher of the acid, (3 to 5%), and the reverse for soft material. Wash in 50% alcohol for several hours. Transfer to ammoniacal alcohol, (96% alcohol, 10 parts, ammonia 1 part) for 2 days. Wash in several changes of distilled water, until the objects sink. Transfer to pyridin for 1 to 2 days. Keep in running water for 12 to 24 hours and pass thru several changes of distilled water. Treat with 2% $AgNO_3$ in incubator at 37° to 38°, for 4 to 10 days, depending on the size of the material. Rinse in distilled water and reduce for 24 hours in: pyrogalic acid, 1 part; formalin, (neutral), 10 parts; distilled water, 100 parts.

Dehydrate in alcohols; (do not leave for over 1½ hours in 70% alcohol). Embed in paraffin, celloidin or both.

The axones of the peripheral nerve fibers, neurofibrils, ganglionic cells, the termination of nerves in muscles, and epithelia, are all well impregnated, showing less shrinkage than by the usual methods.

FOSHAY, LEE. A new method for staining *Bacterium tularense* in tissue sections. J. Lab. & Clin. Med., **17**, 193–195. 1931.

The following stain has given excellent results in revealing *Bacterium tularense* in tissue sections. Add 10 cc. of saturated aqueous nile blue sulfate (neither source nor dye-content specified), and 6 cc. of a 1% aqeous safranin to 60 cc. of H_2O. Stain paraffin embedded sections over night, wash rapidly, pass thru the alcohols, xylol and mount. Nile blue sulfate can also be used alone (saturated aqueous solution) for 5 to 6 minutes. In this case, however, if the bacteria are present in small groups they may be overlooked from lack of contrast.

Zenker's, Orth's, and Dominici's fluids, ethyl and methyl alcohols, as well as formalin all proved quite satisfactory for fixation and none seemed to influence particularly the tinctorial qualities of the stain. An orange filter is recommended for microscopic examination.

Bact. tularense has been thus demonstrated in various organs of guinea pigs, rabbits, mice, and in a fatal human case. The presence of the organism in the liver of a guinea pig is reported for the first time by this or any other method. Other bacteria occur and show up very nicely, such as Pneumococci and Streptococci in pneumonic lungs.

GEIDIES, HERMANN. Nucplascoll, ein neuer Farbstoff für histologische und botanische Schnittfärbungen. Mikr. f. Naturfreunde, **6**, 378–380. 1928.

Nucplascoll is a modified iron hematoxylin stain, with metachromatic qualities. It has been prepared in Dr. Grübler's (Hollborn, Leipzig), laboratory. The author used it successfully on paraffin sections of Zenker, Bouin, and Flemming formalin fixed material in a 2% aqueous solution, allowing it to act 30 to 40 minutes. After staining

the sections were rinsed in H_2O, passed thru 96% alcohol, dehydrated, and mounted in balsam.

Nucplascoll is an excellent nuclear stain, by no means inferior to iron hematoxylin. Microscopic picture: muscle reddish, with dark nuclei; nuclei of the stratum granulosum and basal layer of the epidermis purplish; connective tissue, green; blood corpuscles, light red; gland colloid, transparently pink; cartilage, bluish violet; developing bone, pale violet to greenish.

Plant material seems to require longer staining, and there is no metachromasy as in animal sections. As far as chromatin is concerned, it is very good indeed. The cell walls are slightly colored.

The dry product should be kept well corked and protected from the sun. The sharp and clear chromatin presentation, the differential staining, the ease and quickness of the process make it a valuable histologic stain.

GELLHORN, E. Vital staining and permeability. II Communication. Protoplasma, **12**, 66–78. 1931.

Studies on permeability were conducted by the direct method of vital staining. Fertilized and unfertilized eggs of the sea urchin (*Strongylocentrotus purpuratus*) served for material. Three acid dyes (erythrosin, eosin, and fluorescein) and two basic dyes (methylene blue, and methyl violet) were used; but no information as to the source of exact chemical nature of these dyes is given. Observations made at constant pH-values gave the following results: 1, Ca decreases, Na and Mg increase the permeability of the surface layer of the sea urchin eggs to dye stuffs. The effect of Mg is stronger than that of Na or of other salts with monovalent cations. 2. The increase in permeability due to NaCl or $MgCl_2$ can be reduced or suppressed completely by the addition of $CaCl_2$. 3, The antagonistic effect of $SrCl_2$ and BCl_2 is very slight, if any, in comparison to that of $CaCl_2$. 4, The changes in permeability (increase in $MgCl_2$ and decrease in $CaCl_2$) are compatible with the integrity of the egg cell. They prevent neither the fertilization of unfertilized eggs exposed to these solutions nor the development of fertilized eggs which were kept in them for a length of time sufficient to undergo marked changes in permeability.

GONARD, A. Il n'y a pas d' "amphinucléole" chez les Spirogyra. Comp. Rend. Soc. Biol., **107**, 923–925. 1931.

The disputed question of chromatin nucleoli in Spirogyra has been solved by using a suitable fixative, namely, 94% alcohol. The clarity of mitoses thus obtained clearly demonstrates the distinct individuality of the chromatin from the nucleolus.

GORDON, H. A simple apparatus for cleaning coverslips. J. Lab. & Clin. Med., **17**, 190–192. 1931.

Preparation of the apparatus: Fit one end of glass cylinder, 2 feet long, 2½ inch diameter, into a Buchner funnel. Seal firmly with mixture of litharge and glycerin. Attach the cylinder to a suitable metal stand. Connect the tapering end of the funnel to an air pipe. Fit upper end of the cylinder with a rubber cork pierced with 2 insertions for glass tubes, (about 10 mm. diam.), bent U-fashioned with one arm of the U short, and one long. The long arm should be outside of the cylinder.

Fill the cylinder to about a quarter of its capacity with soap solution and place the cover glasses in it. Replace the rubber cork and turn on the air current. Allow the air to bubble under continuous agitation for about 10 minutes. Rinse in 4 changes of distilled water, aerating each for few minutes. Drain the water off and replace with 96% alcohol. Let the air bubble thru for 5 to 10 minutes. Transfer the cover glasses to a metal tray on paper towelling and allow to dry in a warm place, well protected from dust.

The double exit tubes are of special advantage where the tap water is soft, for then by connecting one of these tubes to the tap, and discontinuing the air tube, the rinsing may be much simplified. The soap solution should not be so heavy as to be too "bubbly" when aerated.

KERNOHAN, JAMES W. A new modification of Mallory-Heidenhain's differential staining method and adaptation of formalin-fixed material for Mallory's stains. Am. J. Clin. Path., **1**, 399–403. 1931.

To employ Mallory's differential stains on tissue fixed in 10% formalin (especially in neuropathology) proceed as follows:

Wash formalin-fixed tissues in running water or ammonia water for a short time (the latter to prevent the accumulation of "formalin precipitate") Then place for four days in Weigert's primary mordant for myelin sheaths (potassium bichromate, 5 g.; chromium fluoride 2 g.; water 100 cc.) and for two days in Weigert's secondary mordant for myelin sheaths (copper acetate 5 g.; chromium fluoride 2.5 g.; acetic acid, 36% 5cc.; water 100 cc.; formalin 10 cc.). Then embed in paraffin and stain in usual way with Mallory's phosphotungstic acid hematoxylin or other staining methods requiring Zenker's fixation. This differentiates the neuroglia, fibroglia, etc., as satisfactorily and clearly as if the tissue had been fixed in Zenker's solution, even after tissue has been several years in formalin.

This modification also makes available Heidenhain's modification of Mallory's ethyl violet orange G stain after fixation in formalin. The resulting picture is: reddish orange myelin, bright blue connective tissue, and blue or purple axis cylinders.

For the pituitary gland, mordant formalin-fixed or unfixed tissue in Weigert's myelin sheath mordants and apply Mallory-Heidenhain's or azocarmin stain. This shows bright red chromophil cells, pale blue chromophobe cells and deep blue or purple basophil cells.—*M. Sherago*

KRUSZYNSKI, P. Une nouvelle methode pour la detection du chondriome. Bull. d'Histol. Appl., **8**, 207–209. 1931.

The following application of Cajal's uranium nitrate method is offered for the clearer staining of the chondriome. It is based on the property of chromic salts to transform lipoids into insoluble compounds.

Fix 10 to 24 hours in Cajal's solution: uranium nitrate 1 part; formalin (neutral) 15 parts; distilled water 85 parts. Rinse rapidly in distilled water. Treat with 3% aqueous potassium bichromate for 1 to 3 days. Wash, pass thru the alcohols, embed in paraffin.

Stain with Heidenhain's hematoxylin or Altmann's basic fuchsin.

KUNZE, R. Der Einfluss der Wasserstoffionene-Konzentration auf die Vakuolenkontraktion vital-gefärbter Elodea-Zellen. Protoplasma, **12**, 161–166. 1931.

These observations confirm with Weber's (Vakuolenkontraktion vital gefärbter Elodea-Zellen, Protoplasma, **9**), on the contraction of the vacuoles of *Elodea* when transferred to tap water from a solution of neutral red (1:1000) in distilled water. Calcium, present in the tap water has not proved to be the determining factor. Of greater importance is the H-ion concentration of the medium. The penetration into the cell of media with a high p-H value causes precipitation of the stain which previously had colored the cell sap but slightly, and eventually brings about the contraction of the vacuole. This contraction does not take place under similar conditions without neutral red.

In conclusion the author says that the contraction of the vacuole is due to changes of the stain which take place when the solution of a pH-value higher than 7.2 penetrates the stained cell sap.

LI YUAN-PO. Méthode pour mettre en évidence, sur la même coupe, les Bacilles tuberculeux et le calcium. Comp. Rend. Soc. Biol., **106**, 648–649. 1931.

The relation between calcium and the tubercle organism may be studied directly by means of this method, for both are revealed in the same section.

Stain with hemalum for 2 to 5 minutes, differentiate under tap for at least 10 minutes. Stain with Ziehl-Neelson fuchsin for 20 minutes without heat. Differentiate in a saturated solution of sodium bicarbonate in 70% alcohol for 30 to 60 minutes, wash. Counterstain with 5% Kühne's blue for 5 minutes. Differentiate in alcohol with some essence of clove for 5 to 10 minutes. Dehydrate, pass thru the xylols and mount. Unfortunately nothing is stated as to the fixative used, the source of dyes, nor are the exact staining formulae given.

The use of an alkaline differentiating agent is indispensable for the preservation of calcium, and is based on Schmorl's procedure. Results: bacteria red, calcium bluish violet, tissue grayish blue. Deformed and granulated bacteria may be seen in calcium masses as well as nuclei stained bluish green, entirely different from the protoplasma impregnated with calcium.

PENFIELD, W. A further modification of Del Rio Hortega's method of staining Oligodendroglia. Am. J. Path., **6**, 445–447. 1930.

The method here offered proved particularly useful for staining the oligodendroglia of the retina, nerve head and optic nerves. Microglia also stained with various success by this method.

Fix tissues for 1 to 2 days in: commercial formalin 20 cc.; urea 4 g.; potassium iodide 6 g.; doubly distilled water 80 cc. Cut sections of about 15 μ on frozen microtome and transfer to distilled water. Wash in 2 dishes of distilled water, the first containing 10 drops of ammonia. Stain from one minute to 1½ hours in del Rio Hortega's solution (10% solution of silver nitrate, Merck, 5 cc.; 5% solution of sodium carbonate 20 cc., add ammonium hydroxide drop by drop, until the precipitate will disappear, stirring constantly, filter and keep in dark bottle). Wash in 60% alcohol, quickly, but thoroly without folding the sections.

Reduce in 1% formalin and wash in distilled water. Tone 10 to 15 minutes with gold chloride (gold chloride, yellow, 1g. distilled water 500 cc.) or until they become grey-purple in color. Fix in 5% hyposulfite of soda, 30 seconds or more, until the sections are flexible. Wash in water. Dehydrate in alcohols, clear in carbol-xylol-creosote, (phenol 10 cc., creosote 10 cc., xylol 80 cc.) and mount in balsam.

URECHIA, C. I. & NAGY, J. Méthode rapide et sure pur la coloration du tissu conjonctif dans le tissu nerveux. Comp. Rend. Soc. Biol., **106**, 498-499. 1931.

By means of this method, (the essential part of which is mordanting with ammonium sulfide) collagen and reticular fibers are clearly revealed.

Fix in formol with ammonium bromide (formalin 15 pts., ammonium bromide 2 pts., distilled water 85 pts.; or preferably, formalin 20 pts., ammonium bromide 5 pts., distilled water 85 pts.), for 3 to 4 days. The fixation may be prolonged to 1 to 2 months without ill effects. Wash in distilled water and mordant in concentrated ammonium sulfide for 15 to 30 minutes in a covered container. Wash the sections in distilled water.

Immerse for 2 to 4 hours, a few sections at a time, in silver carbonate. The silver carbonate solution is to be prepared as follows: Add 5 cc. of 10% silver nitrate to 15 cc. of 5% sodium carbonate. A precipitate forms. Withdraw some of the upper layer and wash twice with distilled water. Dissolve the precipitate by adding concentrated ammonia, drop by drop, and fill with distilled water to 20 cc. Filter the solution before use.

Wash rapidly in 2 containers with distilled water and alcohol, equal parts. Reduce in 1% formol.

Eventually the sections may be passed, as in the Bielschovsky method, thru gold chloride and may be fixed with hyposulfite. Dehydrate, clear in creosote oil and mount in balsam.

VARRELMAN, F. A. Paper apron to prevent curling of microtome sections. Science, **74**, 20. 1931.

A piece of wet tissue paper, preferably dark colored, narrower than the knifeward edge of the block and longer, is placed on the paraffin block. The cut section with the paper will rest flat on the knife with the overlapped edge in such position that the paper, with section adhering, can be removed with forceps or scalpel and placed on a wet slide where the section can be oriented and the paper removed leaving the section in place. The process works more easily with a sliding microtome than with a rotary. (Author abstract.)

WALLACE, H. M. A Stain for fibrin, Gram-positive bacteria, and basal bodies in tissues. Science, **74**, 369–370. 1931.

This is a modification of the Weigert and Gram-Weigert methods, by means of which Gram-positive bacteria are stained and a good tissue stain is obtained at the same time. It has been used for the study of fibrin and pneumococci and is valuable for the determination of intra-cellular bacteria. Other Gram-positive bacteria will also stain successfully.

Fix in Zenker's fluid or Zenker's formalin, (adding 10 cc. of 10% formalin to 90 cc. of the fluid). Stain lightly in hematoxylin and wash. (No indication as to the brand of hematoxylin used is given.) Dip in 0.5% aqueous eosin (Grübler's *wasserlich*) for one-half minute and wash quickly. Stain for 2 hours in Weigert's anilin methyl violet, prepared as follows: Sol. 1) absolute alcohol 33 cc.; anilin oil 9 cc.; methyl violet in excess; Sol. 2) sat. aqueous solution of methyl violet (Grübler's 6B, only); employ 1 part of sol. 1 to 9 parts of sol. 2, and do not mix until few days before use, because the mixture does not keep over 10 days. It acts best when from 3 to 8 days old. Wash and transfer to Lugol's solution for 10 to 15 minutes and wash again. Blot thoroly with filter paper and differentiate in a mixture of 1 part of anilin oil to 2 parts of xylol. Pass thru 2 changes of xylol and mount.

The fibrin, Gram-positive bacteria, and ciliary basal bodies are stained a deep purple, nuclei are dark blue, blood corpuscles usually pale blue, altho at times they remain pink.